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EMBRYO VACCINATION AGAINST *EIMERIA TENELLA* AND *E. ACERVULINA* INFECTIONS USING RECOMBINANT PROTEINS AND CYTOKINE ADJUVANTS

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ABSTRACT: Avian coccidiosis is an intestinal disease caused by protozoa of the genus *Eimeria*. To investigate the potential of recombinant protein vaccines to control coccidiosis, we cloned 2 *Eimeria* sp. genes (EtMIC2 and 3-1E), expressed and purified their encoded proteins, and determined the efficacy of in ovo immunization to protect against *Eimeria* infections. Immunogen-specific serum antibody titers, parasite fecal shedding, and body weight gains were measured as parameters of disease. When administered alone, the recombinant EtMIC2 gene product induced significantly higher antibody responses, lower oocyst fecal shedding, and increased weight gains compared with nonvaccinated controls following infection with *E. tenella*. Combined embryo immunization with the EtMIC2 protein plus chicken cytokine or chemokine genes demonstrated that all 3 parameters of vaccination were improved compared with those of EtMIC2 alone. In particular, covaccination with EtMIC2 plus interleukin (IL)-8, IL-16, transforming growth factor-β4, or lymphotactin significantly decreased oocyst shedding and improved weight gains beyond those achieved by EtMIC2 alone. Finally, individual vaccination with either EtMIC2 or 3-1E stimulated protection against infection by the heterologous parasite *E. acervulina*. Taken together, these results indicate that in ovo vaccination with the EtMIC2 protein plus cytokine/chemokine genes may be an effective method to control coccidiosis.

Infection of poultry by the protozoan parasite *Eimeria* sp. is a serious problem worldwide. Parasites ingested during feeding localize, develop, and reproduce in the intestinal tract, destroying the mucosal barrier and underlying tissues. As a result, feed conversion is reduced and body weight gain is impaired, leading to economic losses for the egg and broiler production industries. Anticoccidial drugs and, to a lesser extent, live and attenuated parasite vaccines, are the primary methods of disease control (Vermeulen et al., 2001; Chapman et al., 2002; Williams, 2002; Lillehoj et al., 2004). However, use of anticoccidials has led to drug resistance, and live vaccines may not be effective against antigenic field variants. Recombinant coccidial vaccines offer an alternative approach, but are limited in their ability to confer cross-immunity to the 7 different *Eimeria* species that infect chickens.

A variety of experimental approaches have been pursued in an attempt to improve the efficacy of recombinant vaccines including, for example, identification of proteins containing shared immunogenic epitopes among the various *Eimeria* species (Lillehoj et al., 2000). Alternatively, coadministration of multiple recombinant immunogens to effectively cover the antigenic repertoire of infective parasites has yet to be tested in the field. Vaccination in the presence of adjuvants (Lillehoj et al., 2004) or providing multiple booster administrations has provided some level of increased protective immunity, but may not represent a cost-effective alternative to the poultry industry. Rather, from a commercial perspective, single event vaccination with a low-cost formulation and utilizing existing technologies constitutes the ideal scenario.

In the current study, we examined the induction of protective immunity to coccidiosis following vaccination at the embryonic stage of development, a technique employed by the poultry industry for more than 20 yr (Johnston et al., 1997; Ricks et al., 1999). We compared the efficacy of 2 different recombinant polypeptide vaccines, EtMIC2, derived from a protein found in coccidia micronemes and involved in host cell attachment and penetration (Tomley et al., 1996); and 3-1E, a parasite surface protein highly conserved among various life cycle stages and species of *Eimeria* (Lillehoj et al., 2000; Song et al., 2000). Additionally, the adjuvant effects of cloned chicken cytokine and chemokine genes on recombinant protein vaccination were determined.

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MATERIALS AND METHODS

Cloning of the EtMIC2 and 3-1E genes

Cloning of the 3-1E gene has been described (Lillehoj et al., 2000; Song et al., 2000). To clone the EtMIC2 gene, *E. tenella* sporulated oocysts were excysted to sporozoites, washed with PBS, and lysed with 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium lauryl sarcosinate, and 0.1 M β-mercaptoethanol. Messenger RNA was purified on an oligo(dT) column (FastTrack 2.0 mRNA Isolation Kit, Invitrogen, Carlsbad, California) and used as a template for cDNA synthesis (cDNA Synthesis Kit, Takara Bio, Shiga, Japan). EtMIC2 cDNA was amplified by PCR using the following primers: forward, 5′-CTTTGTATTCACATTCAAAATGGCTCG-3′; reverse, 5′-CGTCACTCTGCTTGAACCTCTTCC-3′ (GenBank AF111839). Amplification was performed by an initial reaction at 94 C (2 min) followed by 30 cycles at 94 C (1 min), 55 C (2 min), and 72 C (3 min), and a final extension at 72 C (10 min). The 1.1-kb PCR product was gel-purified and subjected to a second round of amplification using the following primers: forward, 5′-GGGAATTCGGCACGAGCTTTGTATTCACATTC-3′; reverse, 5′-GGGTCGACACGTCTTTGCGTCACTCTGCTTGAACC-3′. The amplified fragment was digested with *Eco*RI and *Sal*I, cloned into pBluescript SK(−) phagemid (Stratagene, La Jolla, California), and recombinant EtMIC2-pBL plasmids were confirmed by nucleotide sequence analysis. A *Bam*HI site was inserted upstream of the EtMIC2 coding sequence by PCR using the following primers: forward, 5′-CAGCCGTTAGGATCCGTCCCAGGCG-3; reverse, 5′-GTAATACGACTCACTATAGGGC-3′. Amplicons were digested with *BamH*I and *Sal*I, cloned into pGEX-6p-3 (Amersham Biosciences, Piscataway, New Jersey), and recombinant EtMIC2-pGEX clones were confirmed by sequence analysis. The EtMIC2 coding sequence was subcloned into the *BamH*I/*Sal*I sites of pcDNA3.1 (Invitrogen), transformed into *E. coli* DH5α, and recombinant plasmids were purified (Qiagen, Valencia, California) and quantified spectrophotometrically.

Expression and purification of the EtMIC2 and 3-1E recombinant proteins

Recombinant proteins were expressed and purified as described (Lillehoj et al., 2000; Song et al., 2000). Briefly, the EtMIC2 and 3-1E genes were subcloned in the pMAL plasmid with NH_2-terminal maltose-binding protein tags, expressed in competent *E. coli* DH5α in 1× TY

broth (20 mg/ml tryptone, 10 mg/ml yeast extract, and 10 mg/ml NaCl) containing 100 μg/ml ampicillin. The bacteria were grown to OD_{600} = 0.5, induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside for 3 hr at 37 C, collected by centrifugation, and disrupted by sonication on ice (Misonix, Farmingdale, New York). Proteins were isolated on an amylose affinity column (New England Biolabs, Beverly, Massachusetts) according to the manufacturer's instructions, digested with Factor Xa to release the EtMIC2 or 3-1E proteins, and repassed through the amylose column to remove maltose-binding protein. Final purity was confirmed by SDS-PAGE and Western hybridization.

Chicken cytokine genes

Complementary DNAs encoding IL-2, IL-6, IL-8, IL-15, IL-16, IL-17, interferon (IFN)-α, IFN-γ, transforming growth factor (TGF)-β4, and lymphotactin (LTN), all cloned in the pcDNA vector, have been described (Song et al., 1997; Choi et al., 1999; Lillehoj et al., 2000; Song et al., 2000; Min et al., 2001; Min and Lillehoj, 2002; Min and Lillehoj, 2004). The pcDNA expression plasmids encoding IL-1 (Weining et al., 1998) and IL-18 (Schneider et al., 2000) were used. All plasmids were purified as described above.

In ovo immunization

Eggs of specific pathogen-free white Leghorn SC inbred chickens (Hy-Vac, Adel, Iowa) were incubated to day 18 of embryonation, candled to select fertile eggs, and injected with the purified recombinant EtMIC2 or 3-1E proteins alone or in combination with cytokine genes as described (Sharma, 1987). Briefly, proteins and plasmid DNAs in 100 μl of sterile PBS (pH 7.4) were injected into the amniotic sac by the entire length of a 3.1-cm 22-gauge needle using an Intelliject instrument (Avitech, Easton, Maryland).

Experimental infections

The wild type strains of *E. tenella* and *E. acervulina*, originally developed at the Animal and Natural Resources Institute (Beltsville, Maryland), were used for oral inoculations. Oocysts were cleaned with 5.25% sodium hypochlorite, washed 3 times with PBS, and enumerated with a hemocytometer. Chickens were infected with 10,000 oocysts as described (Lillehoj and Ruff, 1987) at day 14 posthatching. Body weights were measured at day 1 preinfection and day 5 postinfection (P.I.). Oocyst production and shedding were assessed as described by Dalloul et al. (2003). Briefly, fecal samples from individually caged birds were collected between days 5 and 10 P.I., the fecal material for the entire collection period was processed, 2 samples were taken, and the number of oocysts was counted in duplicate for each sample. The final number of oocysts was calculated using the following formula: [Total number oocysts = oocyst count × dilution factor × (fecal sample volume/counting chamber volume)].

ELISA

Flat-bottom, 96-well microtiter plates (Costar, Boston, Massachusetts) were coated with 100 μl of purified EtMIC2 or 3-1E proteins (10 μg/ml) in 0.1 M sodium carbonate buffer pH 9.6 at 4°C overnight. Wells were blocked with 100 μl of PBS containing 1% bovine serum albumin (BSA) for 1 hr at room temperature and incubated with 100 μl of serum samples (1:4 diluted in triplicate wells) for 2 hr at room temperature. The wells were washed 5 times with PBS containing 0.05% Tween-20 and incubated for 30 min at room temperature with 100 μl of horseradish peroxidase-conjugated anti-chicken IgG diluted 1:16,000 in PBS-1% BSA. The wells were washed 5 times, developed with 100 μl of 0.01% tetramethylbenzidine in 0.05 M phosphate-citrate buffer pH 5.0 for 10 min, followed by 50 μl of 2 N H_2SO_4, and optical densities at 450 nm (OD_{450}) were measured.

Statistical analyses

Each experimental group consisted of 5 replicates. Mean and standard deviation (SD) values for antibody levels and body weight gains were compared using the Tukey-Kramer Multiple Comparisons test. Mean ± SD values for fecal oocyst shedding were compared using the Dunnett Multiple Comparisons test. Differences between means were considered significant at $P < 0.05$.

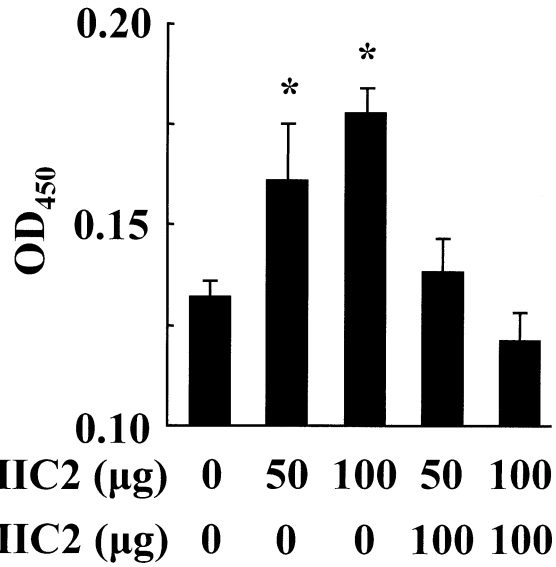

FIGURE 1. Serum antibody levels following vaccination with the EtMIC2 protein. Chickens were vaccinated in ovo with 100 μl PBS (0 μg), or 50 or 100 μg EtMIC2 at day 18 of embryonation (1° immunization). At day 7 posthatching, chickens were nonboosted or boosted (2° immunization) by intraperitoneal injection of 50 or 100 μg EtMIC2. At day 3 postboosting, chickens were bled by cardiac puncture and the levels of anti-EtMIC2 antibodies were measured by ELISA. Each bar represents the mean ± SD (n = 5). Asterisks indicate significantly increased OD_{450} values compared with the nonvaccinated (PBS) control (*$P < 0.05$).

RESULTS

In ovo vaccination with the EtMIC2 recombinant protein stimulates a posthatching serum antibody response

To assess serum antibody levels against the EtMIC2 protein following embryo vaccination, 25 fertile eggs were divided into 5 groups (5/group) and immunized with 100 μl of PBS (group 1) or 50 μg (groups 2 and 4), or 100 μg (groups 3 and 5) of purified EtMIC2 at day 18 of embryonation. At day 7 posthatching, birds were nonboosted (groups 1–3), or boosted by intraperitoneal injection of 50 μg (group 4) or 100 μg (group 5) of EtMIC2. At day 3 postboosting, chickens were bled by cardiac puncture and the levels of anti-EtMIC2 antibodies were measured by ELISA. As shown in Figure 1, EtMIC2 reactive antibodies were detected following primary in ovo vaccination with 50 or 100 μg of recombinant protein. The effect of secondary booster immunizations that antibody production at both doses of immunogen tested was inhibited.

In ovo vaccination with the EtMIC2 recombinant protein stimulates posthatching protective immunity

To assess the effects of in ovo vaccination with the EtMIC2 protein on *E. tenella* infection, 25 eggs were divided into 5 groups (5/group) and vaccinated with 100 μl of PBS (group 1) or 50 μg (groups 2 and 4), or 100 μg (groups 3 and 5) of purified EtMIC2. At day 7 posthatching, birds were nonboosted (groups 1–3) or boosted by an intraperitoneal injection of 50 μg (group 4) or 100 μg (group 5) of EtMIC2. At day 4 postboosting, chickens were orally infected with 10,000 sporulated oocysts of *E. tenella*, and fecal oocyst shedding was determined

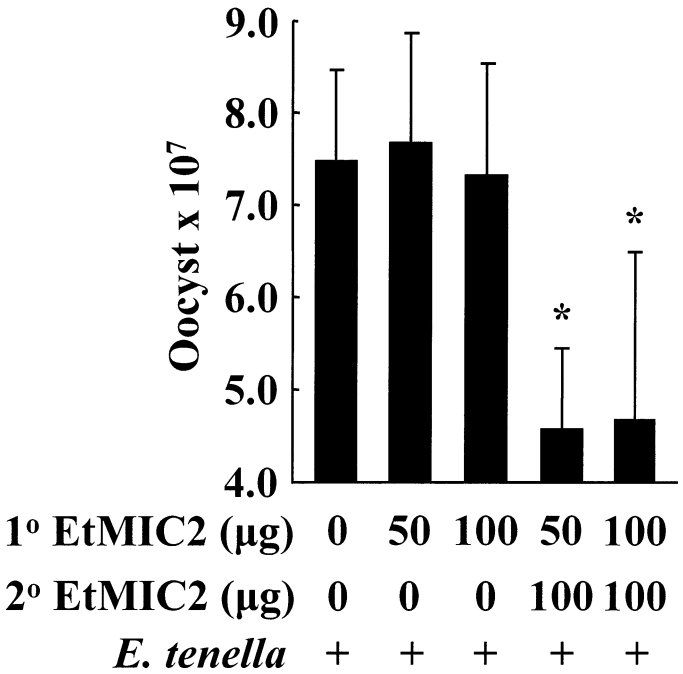

FIGURE 2. Fecal oocyst shedding following vaccination with the EtMIC2 protein. Chickens were vaccinated in ovo with 100 µl PBS (0 µg), or 50 or 100 µg EtMIC2 at day 18 of embryonation (1° immunization). At day 7 posthatching, chickens were nonboosted or boosted (2° immunization) by intraperitoneal injection of 50 or 100 µg EtMIC2. At day 4 postboosting, all birds were orally infected with 10,000 oocysts of *E. tenella*, and cumulative fecal oocyst numbers were determined between 5 and 10 days P.I. Each bar represents the mean ± SD (n = 5). Asterisks indicate significantly decreased oocyst numbers compared with those of nonvaccinated (PBS) controls (*$P < 0.05$).

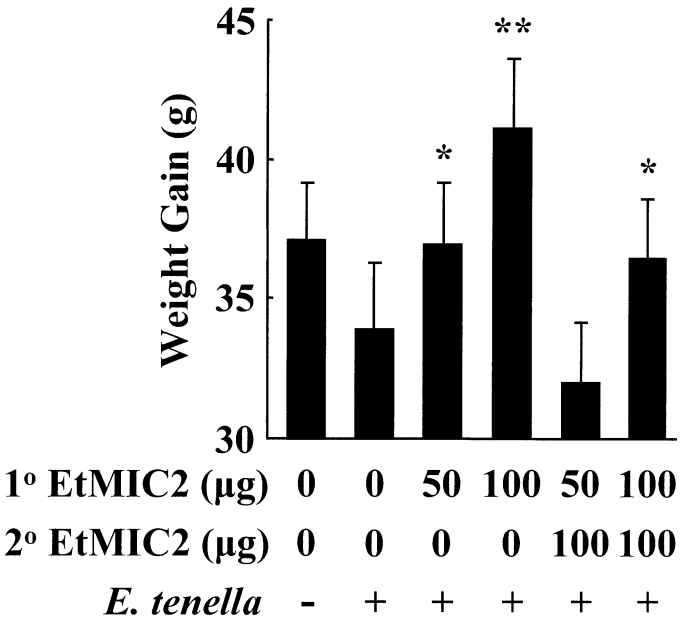

FIGURE 3. Body weight gain following vaccination with the EtMIC2 protein. Chickens were vaccinated in ovo with 100 µl PBS (0 µg), or 50 or 100 µg EtMIC2 at day 18 of embryonation (1° immunization). At day 7 posthatching, chickens were nonboosted or boosted (2° immunization) by intraperitoneal injection of 50 or 100 µg EtMIC2. At day 4 postboosting, birds were either noninfected or orally infected with 10,000 oocysts of *E. tenella*, and body weights were determined at 0 and 6 days P.I. Each bar represents the mean ± SD (n = 5). Asterisks indicate significantly increased weight gain compared with nonvaccinated (PBS) and infected controls (*$P < 0.05$; **$P < 0.01$).

between days 5 and 10 P.I. As shown in Figure 2, only chickens that had received secondary boosting with immunogen displayed significantly decreased oocyst shedding compared with the nonvaccinated controls ($P < 0.05$).

We next evaluated the effects of in ovo vaccination with the EtMIC2 protein on a second parameter of protective immunity: body weight gain following *Eimeria* sp. infection. Thirty eggs were divided into 6 groups (5/group) and injected with PBS (groups 1 and 2) or 50 µg (groups 3 and 5), or 100 µg (groups 4 and 6) of purified EtMIC2. At day 7 posthatching, birds were nonboosted (groups 1–4) or boosted with 50 µg (group 5) or 100 µg (group 6) of EtMIC2. At day 4 postboosting, chickens were noninfected (group 1) or orally infected with 10,000 sporulated oocysts of *E. tenella* (groups 2–6), and weight gains were determined between days 0 and 5 P.I. As shown in Figure 3, with the exception of the 50 µg/100 µg primary/secondary immunization group, all vaccinated birds exhibited significantly increased weight gains compared with the nonvaccinated and infected groups ($P < 0.05$). Taken together, these results indicated that in ovo vaccination with the EtMIC2 recombinant protein induced protection against experimental coccidiosis.

In ovo covaccination with the EtMIC2 recombinant protein plus cytokine/chemokine genes enhances antibody levels and protective immunity compared with EtMIC2 alone

To investigate the ability of chicken cytokine and chemokine genes to potentiate the effects of EtMIC2 protein vaccination, we next analyzed antibody responses, fecal oocyst shedding, and weight gains of infected chickens following in ovo covaccination of EtMIC2 with a panel of 12 cytokines or chemokines. To determine the effects on antibody levels, 70 eggs were divided into 14 groups (5/group) and vaccinated with 100 µl of PBS or 100 µg of the EtMIC2 protein plus 50 µg of pcDNA alone or pcDNA plasmids encoding the IL-1, IL-2, IL-6, IL-8, IL-15, IL-16, IL-17, IL-18, IFN-α, IFN-γ, TGF-β4, or LTN genes. As shown in Figure 4, immunization with EtMIC2 alone significantly increased serum antibody levels compared with those of nonimmunized controls. In addition, covaccination with EtMIC2 plus the IL-18 gene significantly increased antibody levels beyond those obtained with EtMIC2 alone. However, none of the other cytokine or chemokine genes were able to augment EtMIC2 reactive antibody titers compared with what vaccination with the recombinant protein alone could do.

Next, we determined the effects of embryo covaccination with EtMIC2 protein and cytokine/chemokine genes on oocyst shedding following parasite infection. Seventy eggs were divided into 14 groups (5/group) and vaccinated as described above. At day 14 posthatching, all birds were orally infected with 10,000 sporulated oocysts of *E. tenella*, and the numbers of fecal oocysts were determined between days 5 and 10 P.I. Vaccination with EtMIC2 alone significantly decreased oocyst shedding compared with that of the nonvaccinated and infected groups (Fig. 5). In addition, covaccination with EtMIC2 protein plus the IL-2, IL-8, IL-16, IFN-γ, TGF-β4, or LTN genes further decreased oocyst shedding compared with that of EtMIC2 alone ($P < 0.05$). Finally, to assess the effects of in ovo co-

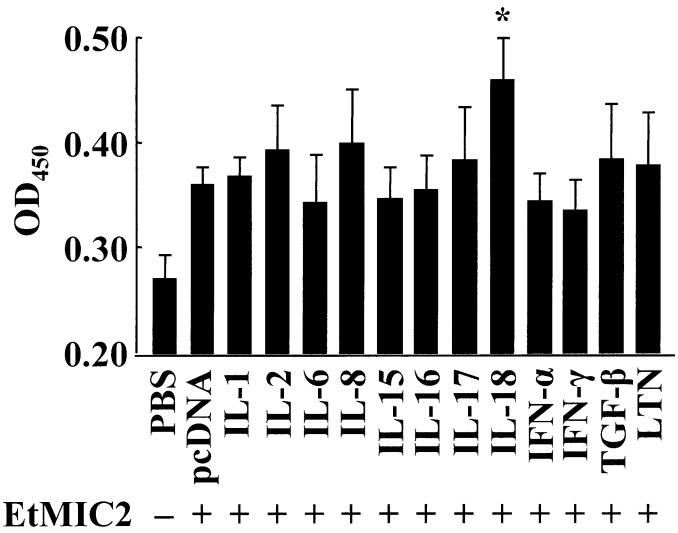

FIGURE 4. Serum antibody levels following covaccination with the EtMIC2 protein plus cytokine genes. Chickens were vaccinated in ovo with 100 μl PBS, or 100 μg EtMIC2 plus 5.0 μg of plasmid DNAs from the pcDNA empty vector or those encoding the indicated cytokine genes. At day 10 posthatching, chickens were bled by cardiac puncture and the levels of anti-EtMIC2 antibodies were measured by ELISA. Each bar represents the mean ± SD (n = 5). Asterisks indicate significantly increased OD_{450} values compared with nonvaccinated (PBS) controls (*$P < 0.05$).

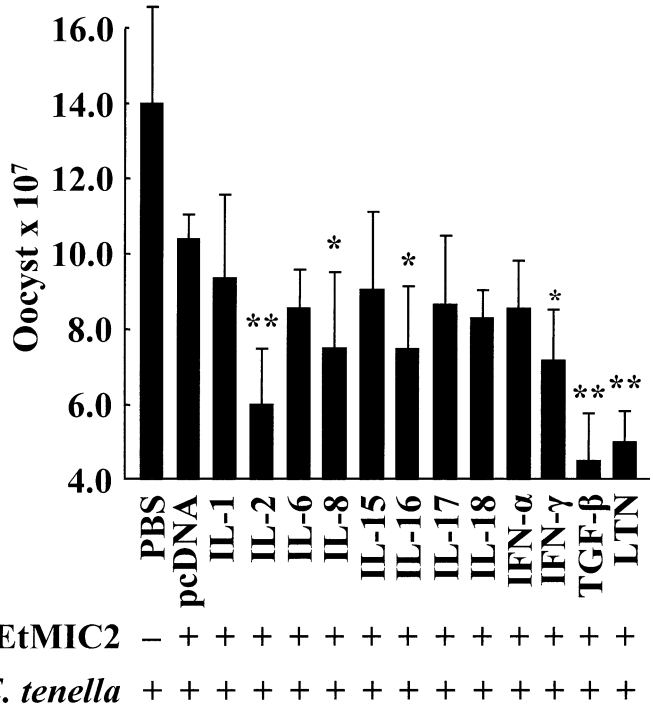

FIGURE 5. Fecal oocyst shedding following covaccination with the EtMIC2 protein plus cytokine genes. Chickens were vaccinated in ovo with 100 μl PBS, or 100 μg EtMIC2 protein plus 5.0 μg of plasmid DNAs from the pcDNA empty vector or those encoding the indicated cytokine genes. At day 14 posthatching, all chickens were orally infected with 10,000 oocysts of *E. tenella*, and cumulative fecal oocyst numbers were determined between 5 and 10 days P.I. Each bar represents the mean ± SD (n = 5). Asterisks indicate significantly decreased oocyst numbers compared with nonvaccinated (PBS) controls (*$P < 0.05$; **$P < 0.01$).

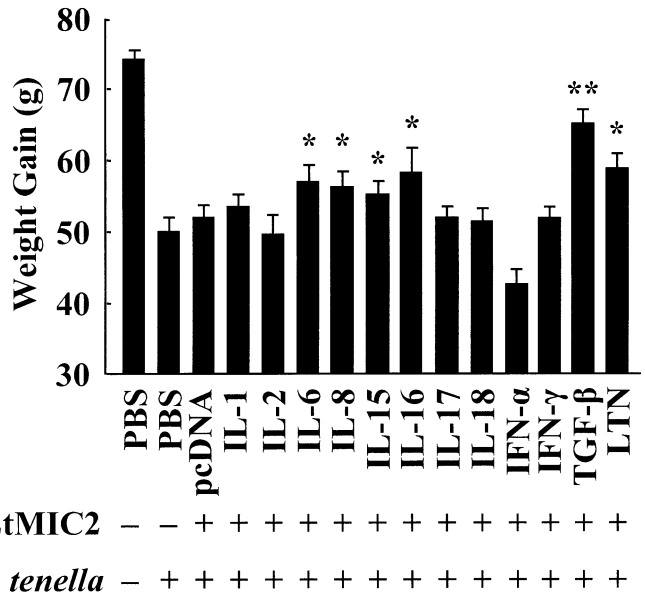

FIGURE 6. Body weight gain following covaccination with the EtMIC2 protein plus cytokine genes. Chickens were vaccinated in ovo with 100 μl PBS, or 100 μg EtMIC2 protein plus 5.0 μg of plasmid DNAs from the pcDNA empty vector or those encoding the indicated cytokine genes. At day 14 posthatching, chickens were either noninfected or orally infected with 10,000 oocysts of *E. tenella*, and body weights were determined at 0 and 6 days P.I. Each bar represents the mean ± SD (n = 5). Asterisks indicate significantly increased weight gain compared with the nonvaccinated (PBS) and infected control (*$P < 0.05$; **$P < 0.01$).

vaccination with EtMIC2 protein and cytokine/chemokine genes on weight gains, 75 eggs were divided into 15 groups (5/group), vaccinated and infected as described above, and body weight gains were determined between days 0 and 5 P.I. Chickens vaccinated with EtMIC2 alone and infected with parasites displayed significantly decreased weight gain (50.3 ± 1.5 g) compared with that in noninfected birds (74.0 ± 1.2 g) (Fig. 6). Covaccination with EtMIC2 plus the IL-6, IL-8, IL-15, IL-16, TGF-β4, or LTN genes significantly increased weight gain greater than that achieved by EtMIC2 alone ($P < 0.05$). In summary, incorporation of the IL-8, IL-16, TGF-β4, or LTN genes into an in ovo vaccine formulation containing the purified EtMIC2 protein increased posthatching protective immunity to *E. tenella* infection as assessed by combined reduction in oocyst shedding and increased body weight gain.

In ovo vaccination with the EtMIC2 recombinant protein stimulates protective immunity against infection by *E. acervulina*

We previously reported the cloning and characterization of an *E. acervulina* gene (3-1E) that encoded a protein conserved among various life cycle stages and species of *Eimeria* (Lillehoj et al., 2000). Therefore, we compared embryo immunization using the EtMIC2 or 3-1E protein vaccines, as well as using a bivalent vaccine containing an equal weight mixture of EtMIC2 and 3-1E to stimulate antibody responses and resistance to challenge infection with *E. acervulina*. In the first experiment, 20 eggs were divided into 4 groups (5/group) and immunized with 100 μl PBS, 100 μg EtMIC2, 100 μg 3-1E, or 50 μg EtMIC2

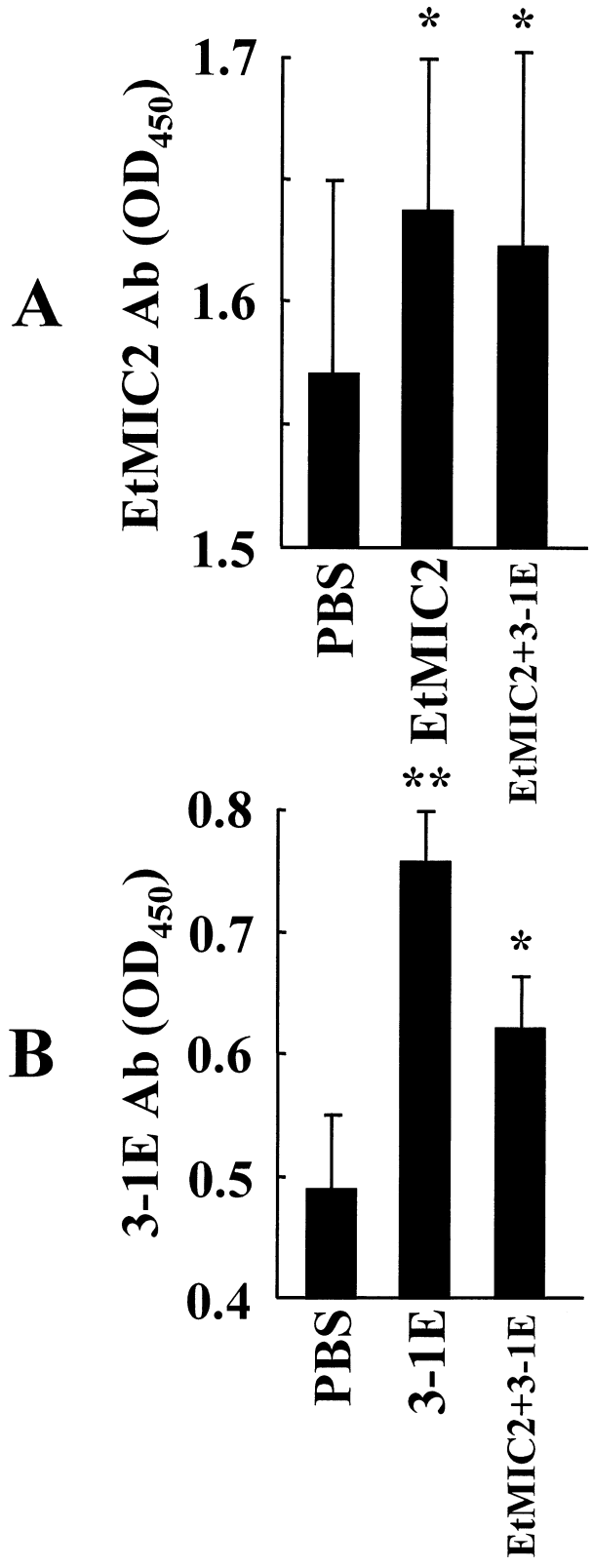

FIGURE 7. Serum antibody levels following vaccination with the EtMIC2, 3-1E, or EtMIC2 plus 3-1E proteins. Chickens were vaccinated in ovo with 100 μl PBS, 100 μg EtMIC2, 100 μg 3-1E, or 50 μg each of EtMIC2 plus 3-1E at day 18 of embryonation. At day 10 posthatching, chickens were bled by cardiac puncture and the levels of anti-EtMIC2 antibodies (**A**) or anti-3-1E antibodies (**B**) were measured by ELISA. Each bar represents the mean ± SD (n = 5). Asterisks indicate significantly increased OD_{450} values compared with those of the nonvaccinated (PBS) control (*$P < 0.05$; **$P < 0.01$).

plus 50 μg 3-1E. At day 10 posthatching, chickens were bled by cardiac puncture and the levels of anti-EtMIC2 and 3-1E antibodies were measured by ELISA. Anti-EtMIC2 antibody titers were significantly increased (Fig. 7A) following vaccination with EtMIC2 alone or EtMIC2 plus 3-1E compared with those of nonimmunized birds ($P < 0.05$). Similarly, anti-3-1E antibodies were significantly greater in the 3-1E only or EtMIC2 plus 3-1E vaccinated groups compared with nonvaccinated controls (Fig. 7B). In the latter case, however, 3-1E-reactive antibody levels were greater in the 3-1E-only group compared with EtMIC2 plus 3-1E vaccinated group.

To determine the effects of in ovo vaccination with EtMIC2, 3-1E, or EtMIC2 plus 3-1E on oocyst shedding following *E. acervulina* infection, 20 eggs were divided into 4 groups (5/group) and vaccinated as described above. At day 14 posthatching, all birds were orally infected with 10,000 sporulated oocysts of *E. acervulina* and the numbers of fecal oocysts were determined between days 5 and 10 P.I. Vaccination with EtMIC2 or 3-1E alone led to significantly decreased oocyst shedding compared with that of nonvaccinated controls ($P < 0.05$) (Fig. 8). In contrast, there was no significant difference between combined immunization with EtMIC2 and 3-1E compared with the nonvaccinated group. In a second vaccination trial, 25 eggs were divided into 5 groups (5/group), vaccinated as above, either noninfected or infected as above, and weight gains were measured. Chickens immunized with EtMIC2 or 3-1E alone exhibited significantly increased weight gains compared with the nonimmunized and infected groups (Fig. 9). Again, however, administration of the EtMIC2/3-1E bivalent vaccine did not improve weight gain following *E. acervulina* challenge infection. Taken together, these results indicate that unlike the individual recombinant proteins, use of a mixture of EtMIC2 and 3-1E failed to stimulate protective immunity to coccidiosis.

DISCUSSION

In this study, we demonstrated that in ovo vaccination with the recombinant EtMIC2 protein induced significantly higher antibody responses, lower oocyst fecal shedding, and increased weight gains following *E. tenella* infection compared with negative controls. Furthermore, combined embryo immunization with the EtMIC2 protein plus chicken cytokine/chemokine genes (IL-8, IL-16, TGF-β4, and LTN) demonstrated enhanced protection compared with vaccination using EtMIC2 alone. Finally, while EtMIC2 was as effective as the 3-1E recombinant *Eimeria* sp. protein in stimulating protective immunity to a heterologous parasite (*E. acervulina*), in ovo covaccination with EtMIC2 plus 3-1E failed to induce protection against oral challenge with this microorganism.

Eimeria species are highly immunogenic, and primary infections can stimulate immunity to subsequent challenge by the homologous parasite. Thus, vaccines may offer excellent alternatives to drugs for controlling coccidiosis in the field (Lillehoj and Lillehoj, 2000; Vermeulen et al., 2001; Brake, 2002; Chapman et al., 2002; Williams, 2002; Lillehoj et al., 2004). How-

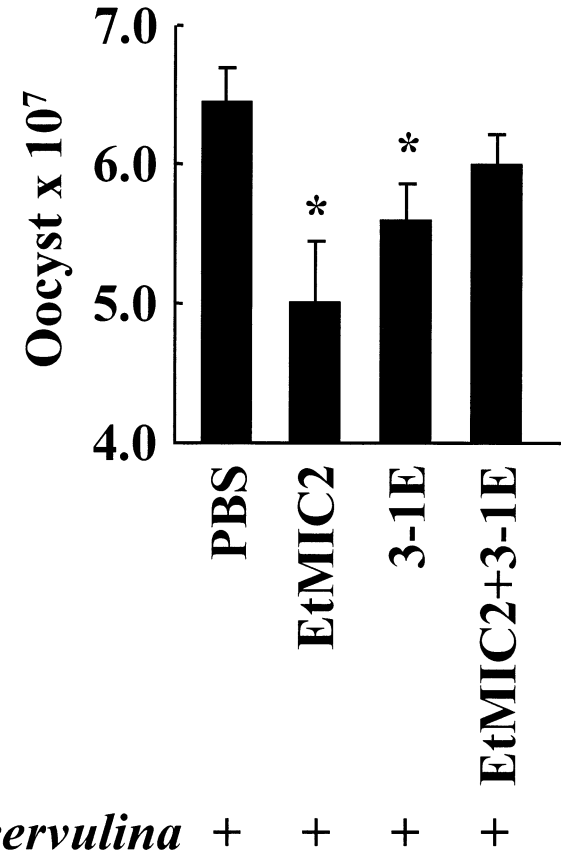

FIGURE 8. Fecal oocyst shedding following vaccination with the EtMIC2, 3-1E, or EtMIC2 plus 3-1E proteins. Chickens were vaccinated in ovo with 100 μl PBS, 100 μg EtMIC2, 100 μg 3-1E, or 50 μg each of EtMIC2 plus 3-1E at day 18 of embryonation. At day 14 posthatching, all chickens were orally infected with 10,000 oocysts of *E. acervulina*, and cumulative fecal oocyst numbers were determined between 5–10 days P.I. Each bar represents the mean ± SD (n = 5). Asterisks indicate significantly decreased oocyst numbers compared with those of the nonvaccinated (PBS) control (*$P < 0.05$; **$P < 0.01$).

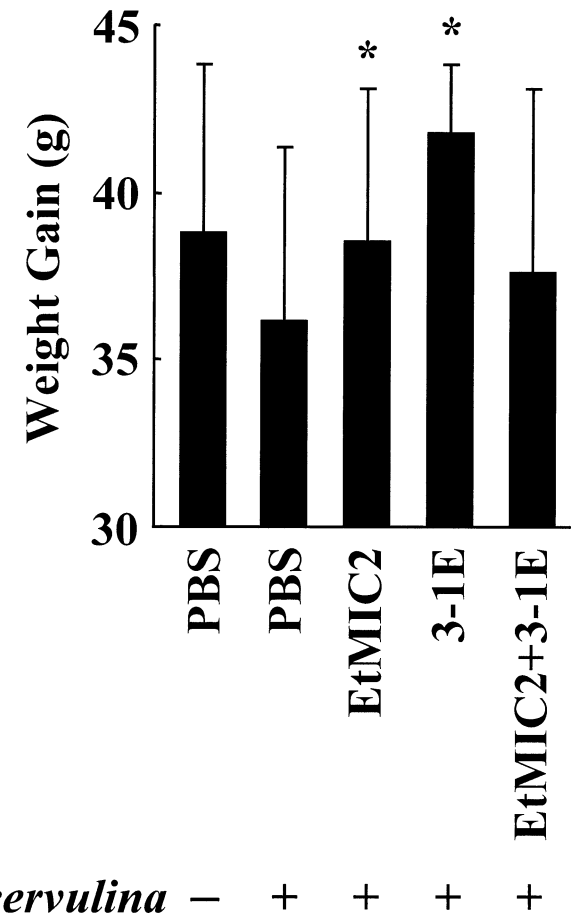

FIGURE 9. Body weight gain following vaccination with the EtMIC2, 3-1E, or EtMIC2 plus 3-1E proteins. Chickens were vaccinated in ovo with 100 μl PBS, 100 μg EtMIC2, 100 μg 3-1E, or 50 μg each of EtMIC2 plus 3-1E at day 18 of embryonation. At day 14 posthatching, all chickens were orally infected with 10,000 oocysts of *E. acervulina*, and body weights were determined at 0 and 6 days P.I. Each bar represents the mean ± SD (n = 5). Asterisks indicate significantly increased weight gain compared with that of the nonvaccinated (PBS) and infected control (*$P < 0.05$).

ever, progress in coccidiosis vaccine development has been hampered by a number of technical and logistical impediments. For example, while many different recombinant *Eimeria* sp. genes and gene products have been isolated and characterized in the past decade, identification of immunogenic, cross-species protective antigens has yet to be reported. Moreover, a method of vaccine delivery that produces optimum resistance to challenge infection has yet to be determined. Recombinant vaccines that have been developed have been delivered orally, subcutaneously, or intramuscularly, eliciting various degrees of protective immunity (Kim et al., 1989; Kopko et al., 2000; Song et al., 2000; Min et al., 2002).

In ovo vaccination against *Eimeria* sp. infection offers an attractive, alternative method of immunization. Embryo vaccination has been used by the poultry industry for more than 20 yr to stimulate posthatch immunity to several economically important avian viruses but has yet to be commercialized for control of *Eimeria* sp. infection (Johnston et al., 1997; Ricks et al., 1999). Weber and Evans (2003) and Weber et al. (2004) reported that immunizing broiler chickens by in ovo injection of sporozoites, sporocysts, or oocysts reduced intestinal lesion scores of chickens subsequent to *E. tenella* infection. In contrast, a study by Watkins et al. (1995) provided no evidence that in ovo administration of *E. maxima* oocysts or sporocysts protected birds from subsequent coccidial challenge. These results were in stark contrast to posthatch inoculation with a single oral dose of *E. maxima* oocysts, which stimulated protective immunity against experimental parasite infection.

To address the issue of cross-species immunity following vaccination with *Eimeria* sp. recombinant proteins, the current study compared immunization with the EtMIC2 and 3-1E gene products for their effects on oocyst shedding and body weight gain following parasite challenge. The EtMIC2 gene was originally cloned from *E. tenella* and shown to encode a microneme adhesin involved in parasite motility and host cell invasion by the parasite (Tomley et al., 1996; Bumstead and Tomley, 2000). The EtMIC2 protein has a 94% homology with the corresponding *E. acervulina* protein, EaMIC2 (data not shown). The 3-1E gene was originally isolated from *E. acervulina* and found to be expressed by sporozoites and merozoites of *E. tenella*, *E. acervulina*, and *E. maxima* (Lillehoj et al., 2000). Similar to

the 3-1E protein, we observed that in ovo administration of EtMIC2 induced significant posthatch protection against *E. acervulina* infection as assessed by both reduced fecal oocyst shedding and enhanced weight gain. Thus, EtMIC2 is similar to CheY-SO7′, a recombinant protein from *E. tenella* that showed cross-protective immunity against *E. acervulina* and *E. maxima* (Crane et al., 1991). Indeed, on the basis of the 2 parameters of disease resistance, vaccination with EtMIC2 was equally as effective against *E. acervulina* as the homologous parasite. Of interest, however, combined immunization with EtMIC2 plus 3-1E failed to confer protection against *E. acervulina*. Previous results clearly showed that 3-1E gene or protein vaccination stimulated resistance to *E. acervulina* infection, although those investigations utilized higher immunogen doses and alternative routes of administration (Lillehoj et al., 2000; Song et al., 2000). Current studies in our laboratory are directed at comparing the optimum vaccination dose and delivery method for EtMIC2, 3-1E, and mixtures of the 2 proteins.

Cytokines as adjuvants enhance immune responses to infectious pathogens (Arai et al., 1993; Lowenthal et al., 1999; Lillehoj et al., 2004). Karaca et al. (1998) described a recombinant fowlpox virus coexpressing chicken type I IFN and Newcastle disease virus hemagglutinin-neuraminidase and fusion genes that offered protective efficacy and humoral responses of chickens following in ovo or posthatch administration of infectious virus. Rautenschlein et al. (1999) reported protection against Newcastle disease virus following embryo vaccination of turkeys with recombinant fowl pox virus constructs containing type I or type II IFN DNA adjuvants. A major research focus of our laboratory has been the identification of chicken cytokines as immunomodulators of *Eimeria* sp. vaccines. For example, recombinant IFN-γ was shown to inhibit *E. tenella* development in vitro and to reduce oocyst production and body weight loss following *E. acervulina* infection in vivo (Lillehoj and Choi, 1998). Subsequently, we showed that subcutaneous injection of the recombinant 3-1E protein, or the 3-1E cDNA, with cDNAs encoding IL-2, IL-15, or IFN-γ, enhanced protective immunity to *E. acervulina* challenge (Lillehoj et al., 2000). Min et al. (2001) confirmed these results and added the IL-1, IL-8, TGF-β4, and LTN genes to the list of cytokine adjuvants that reduced the severity of *E. acervulina* infection when injected subcutaneously into chickens along with the 3-1E gene. In the present study, coadministration of the IL-8, IL-16, TGF-β4, or LTN genes with EtMIC2 protein significantly decreased oocyst production and increased weight gain compared with that of EtMIC2 alone. Thus, the adjuvant effect of chicken cytokine genes involves many different immunomodulators and is effective with different *Eimeria* sp. recombinant proteins. Determination of the most effective immunogen/cytokine combination, the optimal doses of each, and the best route of vaccination will eventually lead to formulation of a novel effective coccidiosis vaccine for future field trials.

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